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PRINCIPAL INVESTIGATOR: Byeong-Chel Lee, PhD

CONTRACTING ORGANIZATION: University of Pittsburgh, Pittsburgh, PA 15213

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14. ABSTRACT How leukemia stem cells gained resistance to radiation and chemotherapy is poorly defined, yet critically determines how leukemia cells tolerate conventional leukemia therapy. Normal hematopoietic stem cells and leukemia initiating cells are known to share many functional properties. Therefore, they are supposed to utilize many common mechanistic pathways for their survival and migration. Using genetically engineered mice we demonstrated the functional roles of P2Y ₁₄ in preserving regenerative capacity by constraining senescence induction and molecular events governing it. Since P2Y ₁₄ is highly expressed in differentiation-resistant leukemia cells, P2Y ₁₄ expression in leukemia cells may also function in modulating the resistance to conventional cancer treatment. Our results strongly suggest that P2Y ₁₄ /UDP-Glc axis plays an important role in stress-induced senescence and motility of HSPCs. As the expression of P2Y ₁₄ is preferentially high in drug-resistant leukemia cells, we anticipate that P2Y ₁₄ /UDP-Glc axis governs the resistance and motility also in leukemia stem/progenitor cells.		

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Introduction

Although conventional therapy temporarily lessens the burden of the disease, a lingering subpopulation of drug- and radiation-resistant leukemia may regenerate. This small subpopulation of drug- and radiation-resistant leukemia is an immediate concern for leukemia patients as this subtype remains the actual cause of morbidity and mortality.

Nucleotides, once recognized as mere sources of energy, are now emerged as key extracellular messengers that regulate diverse aspects of homeostasis in various physiological and pathophysiological conditions (Volonte et al., 2006). Extracellular nucleotides exert their actions through interaction with their cognate receptors, purinergic receptors. Purinergic receptors are classified into P1 and P2 receptors, based on their ligand binding and function (White and Burnstock, 2006). The role of P2 receptors as regulators of hematopoiesis has become more evident in recent years (Di Virgilio et al., 2001b; Sak et al., 2003). A wide variety of P2 receptors are expressed in blood and inflammatory cells, and their physiological significance has been demonstrated (Di Virgilio et al., 2001a).

Recent results defined molecular signatures predicting the drug resistance of leukemia cells (Tagliafico et al., 2006). P2Y₁₄ expression has been shown to be highly upregulated in differentiation-resistant acute myeloid leukemia (AML) cases in 28 freshly isolated AML blast populations, making the P2Y₁₄ gene a prime suspect of incurable leukemia. P2Y₁₄ is also listed as a target gene of the *Wnt3A*, whose aberrant regulation is closely associated with hematological malignancies and several types of other cancers (Nygren et al., 2007). In this report, when leukemia cells are treated with Wnt3A, P2Y₁₄ was the gene most strongly upregulated. More recently, a comprehensive mutational analysis of human cancer identified P2Y₁₄ as one of the candidate cancer genes that is mutated at a significant frequency in a large fraction of colorectal cancers (Sjoblom et al., 2006).

It is believed that a similar set of genes controls both normal and cancer stem cells. Therefore, if the genes expressed by normal stem cells are found to be mutated or used differently in cancer cells, it is very likely that those genes play a role in the development of cancer stem cells. Our preliminary findings demonstrate a novel role for P2Y₁₄ in the response to radiation and chemo reagents, serving as a modifier of cell senescence and cell death, thereby enabling preservation of hematopoietic stem/progenitor cell (HSPC) function. Considering the similarity between normal and leukemic stem cells, our preliminary results lead to the likelihood

that P2Y₁₄ is closely associated not only with maintenance of the normal HSC but also with the drug-resistant leukemia cells.

Bone marrow transplantation (BMT) is a potentially everlasting curative therapy for hematological diseases such as leukemia, lymphoma and various types of immunologic disorders. In the past years, bone marrow cells have been replaced by mobilized peripheral blood stem cells (PBSCs), because PBSCs engraft better than bone marrow-derived HSPCs and allow faster recovery of the white blood cell count. With faster engraftment and reduced risk of posttransplant infection, mobilized cells became a major source of HSPCs for autologous and allogeneic transplantations. Our preliminary findings demonstrate that UDP-Glucose (UDP-Glc), a putative ligand of P2Y₁₄ receptor, has a previously unknown function in mediating HSPC mobilization.

Taken together, we propose that the P2Y₁₄ receptor is an important local regulatory molecule that leads to both normal and leukemic stem cell migration and quiescence and the manipulation of P2Y₁₄/UDP-Glc signaling axis may modulate the susceptibility of normal and leukemic stem cells to radiation and chemo reagents.

Body

Specific Aim 1: To investigate how P2Y₁₄ signaling axis regulates the quiescence of LSC

Cell cycle quiescence of normal and leukemia stem cells is one of the key features which allow these cells to avoid being killed by conventional cancer therapy. It is hypothesized that leukemic stem cells arise either from normal stem cells or from progenitor cells. In this specific aim, we aimed to test the hypothesis that the activation of P2Y₁₄/UDP-Glc axis regulates cell cycle quiescence of normal and leukemia stem cells. Since quiescent cells are more resistant to conventional cancer treatments such as chemo-and radiation therapy, if our hypothesis turns out to be correct, by manipulating P2Y₁₄/UDP-Glc axis, we may modulate the drug sensitivity of leukemic cells. In addition, P2Y₁₄ gene is a signature molecule in differentiation resistant leukemia cells (Tagliafico et al., 2006), highlighting its important translational potential in the treatment of recurrent leukemia. We first tested whether UDP-Glc treatment induces cell cycle quiescence in HSPCs. Indeed, compared with the bone marrow LSK cells isolated from G-CSF-treated mice (hatched bars in Figure 1), the bone marrow LSK cells isolated from UDP-Glc

treated mice (black bars in Figure 1) contained a significantly higher proportion of cells in G0 and lower proportion in the G1, S, and G2/M phases.

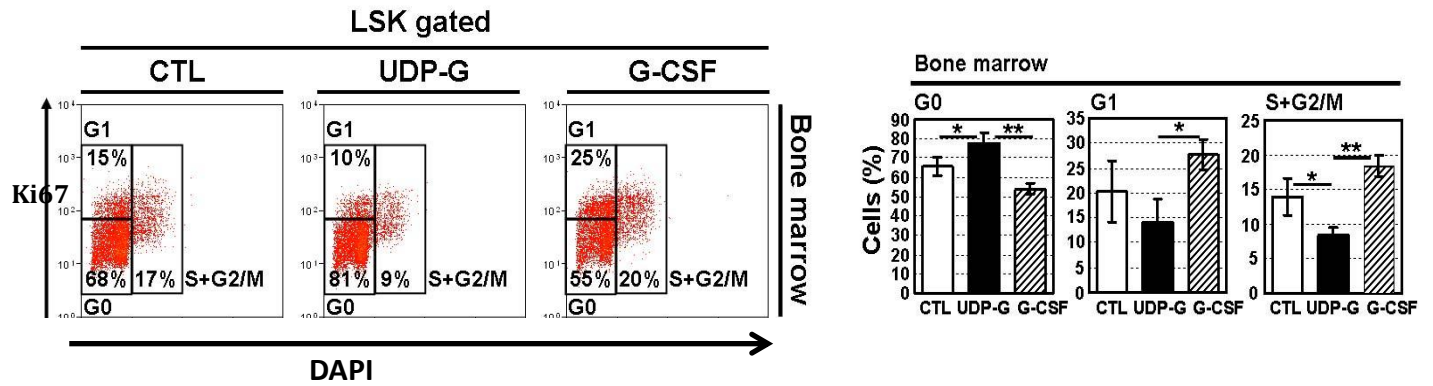


Figure 1. The effect of UDP-Glc on cell cycle status of HSPCs

Mice were injected once daily with UDP-Glc or PBS (CTL) for 6 days. The bone marrow samples were pooled from each group ($n > 4$) and stained for Ki67 and DAPI. LSK cells were pregated and further analyzed for their cell cycle status. Left: The fraction of cells in the respective cell cycle phases is indicated in percent. Right: The data shown are the mean \pm s.d. of three independent experiments with four to five mice per group.

In our previous annual reports, we demonstrated that *P2ry14* deficiency confers hypersusceptibility to IR and chemotherapy drug, such as 5-fluorouracil (5-FU). Taken together, our results suggest that $P2Y_{14}$ /UDP-Glc plays a role in drug resistance of stem progenitor cells in part by regulating their cell cycle progression.

Based on these results, we aim to further investigate whether $P2Y_{14}$ /UDP-Glc signaling axis regulates the cell cycle status of immature leukemia cells such as KG1a or KG1 cells. Our preliminary data showed that these leukemic cell lines express $P2Y_{14}$ on their cell surface. Simultaneously, these leukemic cell lines will be transplanted into NOD-*scid* *IL2R γ ^{null}* mice. After 3-4 weeks of transplantation, the engraftment of donor cells will be quantified by flow cytometry using anti-human CD45 antibody. After we confirm the donor cell engraftment, the recipient animals will be treated with UDP-Glc followed by the cell cycle analysis of donor leukemia cells.

Specific Aim 2: To investigate functional correlation between P2Y₁₄ signaling and therapy-resistant leukemia

In the previous reports, we showed that p38MAPK is hyperactivated in P2Y₁₄ deficient LSK and SLAM LSK cells following radiation. p38 MAPK, Erk and JNK are well-known G-protein coupled receptor downstream molecules. As we reported in our previous report, inhibiting p38 MAPK activity lessened IR-induced HSPC senescence. To investigate whether the hypersensitivity of P2Y₁₄ deficient LSK and SLAM LSK cells against IR stress is indeed due to the absence of P2Y₁₄ receptor, we attempted to block P2Y₁₄ signaling pathway using pertussis toxin (PTX). P2Y₁₄ receptor couples to G α -subunits of the Gi family of heterotrimeric G proteins. Since PTX will eliminate the activity of all members of the G α i/o protein including P2Y₁₄-mediated activation, P2Y₁₄ KO cells wouldn't be distinguishable from their WT counterparts in their responses to IR-induced senescence. While our results showed that PTX treatment induced cellular senescence both in WT and KO HSPCs, the difference between WT and KO did not exist anymore, suggesting that the differential sensitivity of P2Y₁₄ KO HSPCs against IR-induced senescence is largely due to the P2Y₁₄-mediated G α i/o stimulation (Figure 2).

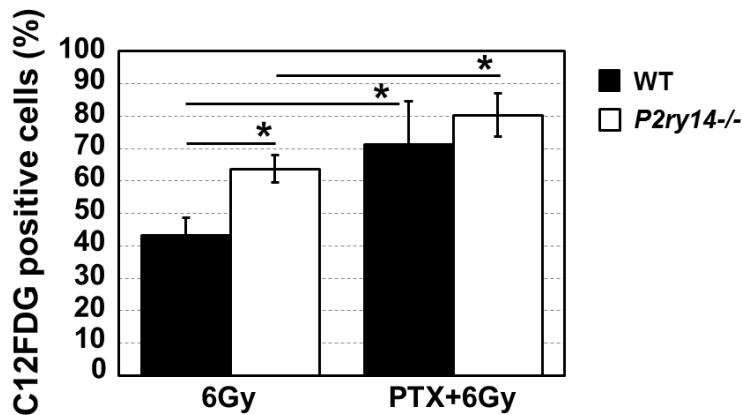


Figure 2. The difference in IR-induced senescence between WT and KO HSPCs disappears upon PTX treatment.

WT and KO mice were injected i.v. with PTX (0.8 μ g/mouse). Thirty minutes after PTX injection, the mice were subjected to 6 Gy TBI, and then the bone marrow cells were transplanted into lethally irradiated recipient mice. Recipient animals were sacrificed 10-12 days after transplantation and the donor-derived LSK cells were analyzed for the extent of senescence.

Interestingly, when normal mouse thymocytes were exposed to various doses of irradiation, there was dose-dependent increase of cell surface expression of P2Y₁₄ receptor, suggesting a potential causal relationship between P2Y₁₄ receptor expression and radiation response (Figure 3).

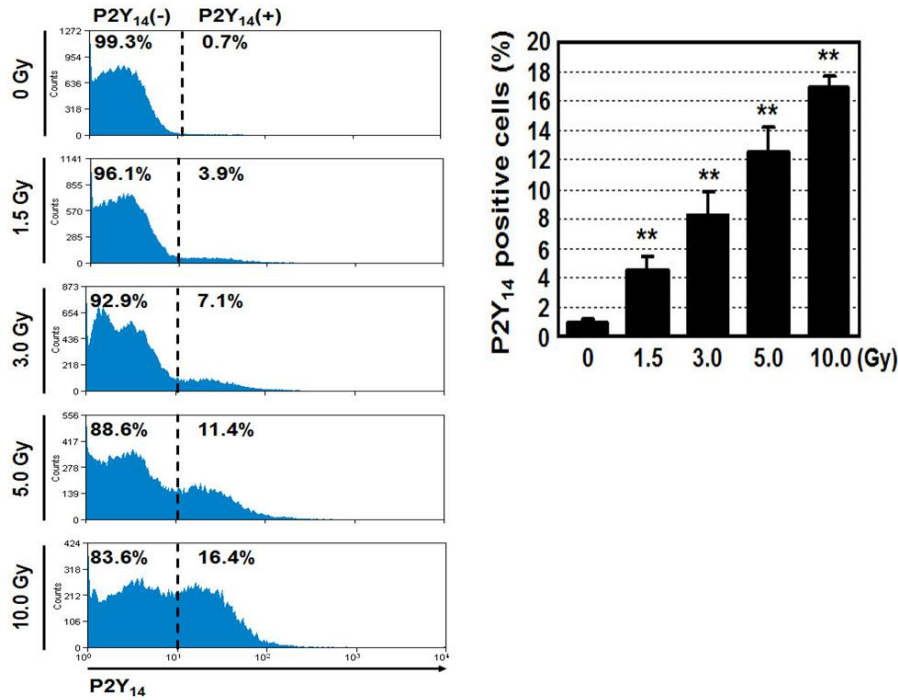


Figure 3. Dose-dependent increase of P2Y₁₄ receptor upon radiation

Thymocytes were prepared from 4-6 week-old wild type mice and exposed in vitro to different doses of γ -irradiation as indicated. Cells were stained with biotinylated anti-P2Y₁₄ antibody and APC-conjugated streptavidin. Percentages of gated cell populations are indicated. The accompanying graphs show the percentage of P2Y₁₄⁺ thymic cells.

As our results indicate that P2Y₁₄/UDP-Glc signaling axis can alter the radiation resistance of cells, we will further investigate the molecular pathways involved in radiation induced- apoptosis or -senescence such as p53, p21, p16 and p38 MAPK. As proposed in Specific Aim1, we will utilize human leukemia cell lines to examine if P2Y₁₄/UDP-Glc differentially regulate p53, p21 and p16 thus resulting in different levels of cell death or senescence upon irradiation. If the results suggest that UDP-Glc modulates radiation-induced cell death and senescence, we will transplant the human leukemia cell lines to establish xenograft models, and then examine if UDP-Glc administration can regulate the resistance of engrafted leukemia cells against chemo- or radiation-induced cell death or senescence.

Specific Aim 3: Examine whether the activation of P2Y₁₄/UDP-Glucose signaling axis mobilizes leukemic stem cells from recipient's bone marrow.

Mobilized HSPCs are a major source of peripheral stem cell transplantation (PSCT) for leukemia patients. With faster engraftment and reduced risk of posttransplant infection, mobilized HSPCs are now more commonly used as stem cell sources. In our previous progress reports we showed the ability of UDP-Glc to mobilize CFU-Cs and LSKs into the blood circulation. We continued our attempts to unravel mechanisms underlying UDP-Glc-mediated HSPC mobilization.

Controversy still exists regarding the role of osteoclasts in regulating HSPC mobilization (Calvi et al., 2003; Kollet et al., 2006; Miyamoto et al., 2011; Takamatsu et al., 1998) raising a question as to whether osteoclasts indeed play an essential role in UDP-Glc-mediated HSPC mobilization. To address this question, we first utilized the osteopetrotic (*op/op*) mouse model. Mice homozygous for the *op* mutation exhibit a severe deficiency of osteoclasts so that this strain can serve as a model to investigate the role of osteoclasts in UDP-Glc-mediated HSPC mobilization (Yoshida et al., 1990). Administration of UDP-Glc into littermate control mice (CTL; *+/op*) induced osteoclastogenesis and promoted the mobilization of LSK cells and SLAM LSK cells (Figure 4). However, *op/op* mice given the same treatment showed no changes in osteoclastogenesis and failed to show a statistically significant increase in the number of peripheral LSK and SLAM LSK cells (Figure 4). These results suggest that osteoclasts play an important role in the regulation of UDP-Glc-mediated HSPC mobilization.

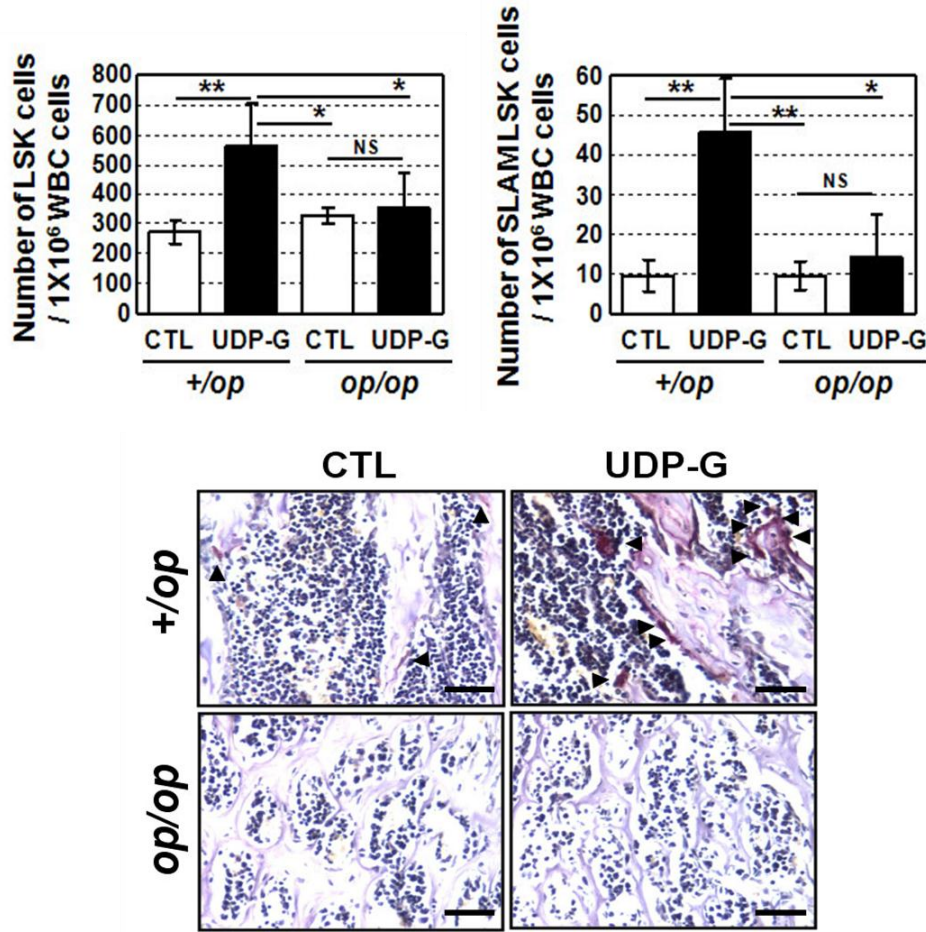


Figure 4. Role of osteoclasts in UDP-Glc-mediated HSPC mobilization

Osteopetrotic (*op/op*) mutant mice (n=6 per treatment group) and their littermate wild-type controls (n=10 per treatment group) were treated with either vehicle (CTL) or UDP-Glc. Mice were treated with UDP-Glc in the same dosage and schedule as described above. HSPC mobilization was assessed by measuring the numbers of LSK (upper left panel) and SLAM LSK (upper right panel) cells in peripheral blood. Mice were individually analyzed for each group, and the mean \pm SD is shown. * $p < 0.05$ and ** $p < 0.01$. Representative images of TRAP staining from each group are shown (bottom panels).

To further study the impact of osteoblasts/osteoclasts in UDP-Glc-mediated HSPC mobilization, P2X₇ deficient mice were analyzed. Deficiency of P2X₇ in the mouse results in impaired bone formation and excessive bone resorption (Ke et al., 2003). In accordance with this finding, a significantly increased numbers of osteoclasts were detected in non-treated P2X₇ KO mice (Figure 5). UDP-Glc did not lead to a further noticeable increase in osteoclast activity in

P2X₇ KO mice. Similarly, UDP-Glc-treated P2X₇ KO mice showed no significant increase in the number of peripheral LSK cells, compared to the vehicle-treated P2X₇ KO mice (Figure 5, upper left). There was a trend towards moderately increased numbers of SLAM LSK cells (~1.9 fold) in the blood of UDP-Glc-injected P2X₇ KO mice. However, this did not reach statistical significance. Notably, steady-state basal levels of circulating LSK cells were elevated in P2X₇ KO mice compared to those in WT mice (Figure 5, upper left), suggesting the possibility that P2X₇ deficiency may lead to constitutive LSK cell mobilization in part through increased osteoclast activity. Taken together, these results suggest a potential role of osteoclasts in UDP-Glc-induced HSPC mobilization.

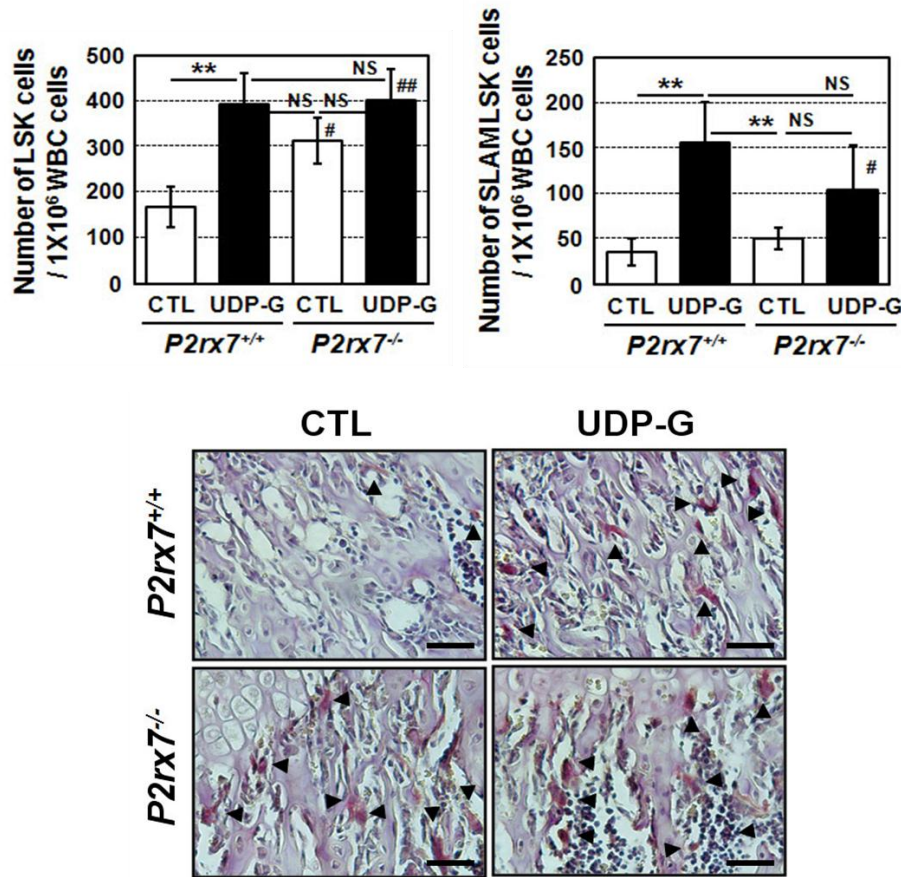


Figure 5. P2X₇ (*P2rx7*^{-/-}) KO and their littermate wild-type (*P2rx7*^{+/+}) controls were treated with either vehicle (CTL) or UDP-Glc as described above. HSPC mobilization was assessed as described in Figure 4. Mice were individually analyzed for each group (n = 10/treatment group), and the mean ± SD is shown. **p* < 0.05 and ** *p* < 0.01. Representative images of TRAP staining from each group are shown (bottom panels). Osteoclasts are already present at high numbers at about six weeks of age in *P2rx7*^{-/-} KO mouse. Data were obtained from 6-8 weeks old animals.

Our findings have important clinical implications in designing new mobilization strategies to improve the efficiency and outcome of autologous and allogeneic peripheral blood stem cell transplantation in leukemia patients. Based on the results from above, we will investigate if UDP-Glc plays a regulatory role in the egress of leukemia cells from the bone marrow. For this purpose, we will establish xenograft model as described above. UDP-Glc will be administered and the number and frequency of human specific CD45+ cells in the peripheral blood of recipient mice will be assessed. Once we detect human leukemia cells in recipient animal's peripheral blood, we will further analyze the absolute number of CD34+ 38- cells in human cell-gated population (ex: hCD45+, CD34+38-)

Key Research Accomplishments

1. P2Y₁₄ functions in bone marrow to preserve hematopoietic stem/progenitor cells from premature senescence and cell death induced by genotoxic stress.
2. We identified potential mechanisms by which P2Y₁₄ signaling axis mediates radiation and chemo reagent resistance.
3. We demonstrate that UDP-Glucose has a previously unknown function in mediating HSPC mobilization.
4. Differential sensitivity of P2Y₁₄ KO HSPCs against IR-induced senescence is largely due to the P2Y₁₄-mediated Gαi/o stimulation.
5. Osteoclasts play an important role in the regulation of UDP-Glc-mediated HSPC mobilization.

Reportable Outcomes

Cho JS, Shen H, Hui Y, Cheng T, Lee SB, Lee BC. 2011. Ewing's Sarcoma Gene EWS regulates Hematopoietic Stem Cell Senescence. *Blood*, 117:1156-66.

Cho JS, Kook SH, Robinson A, Niedernhofer L, Lee BC. Endogenous DNA damage drives the loss of hematopoietic stem cell number and function via cell autonomous and non-autonomous mechanisms. 2013, *Stem Cells*, 31(3):511-25

Kook SH, Cho JS, Morrison A, Wiener E, Lee SB, Scadden D, Lee BC. The purinergic P2Y₁₄ receptor axis is a molecular determinant for organism survival under *in utero* radiation toxicity. 2013, *Cell Death & Disease*, **Accepted**

Kook SH, Cho JS, Lee BC. A Nucleotide Sugar, UDP-Glucose, is a Novel Mobilizer of Long-Term Repopulating Primitive Hematopoietic Cells. *Journal of Clinical Investigation*, **Accepted**

Conclusion

How leukemia stem cells gained resistance to radiation and chemotherapy is poorly defined, yet critically determines how leukemia cells tolerate conventional leukemia therapy. Normal hematopoietic stem cells and leukemia initiating cells are known to share many functional properties. Therefore, they are supposed to utilize many common mechanistic pathways for their survival and migration. Using genetically engineered mice we demonstrated the functional roles of P2Y₁₄ in preserving regenerative capacity by constraining senescence induction and molecular events governing it. Since P2Y₁₄ is highly expressed in differentiation-resistant leukemia cells, P2Y₁₄ expression in leukemia cells may also function in modulating the resistance to conventional cancer treatment. Our results strongly suggest that P2Y₁₄/UDP-Glc axis plays an important role in stress-induced senescence and motility of HSPCs. As the expression of P2Y₁₄ is preferentially high in drug-resistant leukemia cells, we anticipate that P2Y₁₄/UDP-Glc axis governs the resistance and motility also in leukemia stem/progenitor cells.

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Appendices

N/A